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Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation

(cosuppression/cytosine methylation/epigenetics/*Nicotiana tabacum*/transgenic plant)

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ABSTRACT **Endogenous plant genes or transgenes can be silenced on introduction of homologous gene sequences. Here we document a reporter gene-silencing event in *Nicotiana tabacum* that has a distinctive combination of features—i.e., (*i*) silencing occurs by a posttranscriptional process, (*ii*) silencing correlates with DNA methylation, and (*iii*) this *de novo* methylation is not restricted to cytosines located in the symmetrical motifs CG and CXG.**

When a transgene, containing sequences homologous to an endogenous gene(s), is introduced into plants, expression of both the introduced transgene and the homologous host gene(s) can be suppressed (1, 2). This phenomenon is often referred to as cosuppression (for reviews, see refs. 3–6). Similarly, the introduction of multiple copies of a transgene into a plant genome sometimes results in highly reduced expression levels of these genes (7–11). Genetic and molecular analyses of the transgene silencing phenomena indicate that silencing is meiotically reversible (8) or is transmitted stably through several generations (12, 13). Gene silencing was observed to clearly correlate with DNA methylation in several described cases (8, 11, 12, 14), but not to correlate in other cases (10, 15, 16). Transgene-induced gene silencing phenomena also differ with respect to the level at which silencing occurs: in some systems, silencing occurs at the transcription level (12, 17, 18), whereas in other cases silencing is due to a posttranscriptional process (19–21). The cellular mechanism(s) responsible for these various cases of transgene silencing remains unknown (for review, see refs. 3–6). There is increased evidence that beside unknown factor(s), the copy number and configuration of the integrated transgene, the levels of transgene transcripts, and environmental and developmental factors are involved in establishing gene silencing (8, 11, 20, 22, 23).

While studying 3′ end formation in plant cells, tobacco plants were obtained with a transferred DNA (T-DNA) construct harboring a chimeric hygromycin gene, which was used as a selectable marker, and a neomycin test gene under control of the cauliflower mosaic virus 35S promoter (P35S) (24). Expression of the neomycin gene was severely reduced in some primary transformants containing multiple T-DNA loci. One of these plants was analyzed in detail. We show that it represents a case of transgene cosuppression with characteristics previously described for distinct gene silencing events. The transgene silencing is subject to posttranscriptional control and yet is correlated with DNA methylation. This *de novo* methylation is not restricted to cytosines located in the CG or CXG context.

MATERIALS AND METHODS

Plasmids. The T-DNA-derived plant transformation vector pGVCHS(320) harbors two chimeric genes between the T-DNA borders: the neomycin phosphotransferase II-coding sequence (*nptII*) under control of the P35S fused to a 320-bp *Sma* I/*Bam*HI fragment encompassing the polyadenylylation site of the *Antirrhinum majus* chalcone synthase gene (*chs*) and the hygromycin phosphotransferase-coding sequence (*hpt*) under control of the nopaline synthase promoter (Pnos) and 3′-untranslated region (24).

pGEMnpt, pGEMrib, and pGEMrbcS were constructed by subcloning the coding region of the *nptII* gene as a 846-bp *Bgl* II/*Asu* II fragment, *Arabidopsis thaliana* rDNA sequences as a 4.3-kb *Eco*RI fragment (25), and the complete *Nicotiana tabacum* NtSS23 ribulose-1,5-bisphosphate carboxylase gene (*rbcS*) as a 2.4-kb *Hin*dIII fragment (26) into the polylinker of either pGEM-1 or pGEM-2 (Promega).

***Agrobacterium*-Mediated Plant Transformation.** pGVCHS-(320) was mobilized to *Agrobacterium* C58C1Rif^R by triparental mating and cointegrated into the resident pGV2260 virulence plasmid (27). *N. tabacum* var. SR1 was transformed via the leaf disc infection method (28). Transformed plants were selected on medium containing hygromycin at 50 mg per liter. For segregation analysis, seeds were placed on medium containing hygromycin at 25 mg per liter.

RNA/DNA Isolation and Blot Hybridization. Total RNA was isolated from leaves of mature *N. tabacum* plants as described (29). After lithium acetate precipitation, the DNA in the supernatant was isolated by isopropanol precipitation. Total RNA (10 μg per lane) was electrophoresed in a 1.5% agarose/formaldehyde gel and transferred to Hybond-N membranes (Amersham). Blotting and hybridization were done as recommended by the manufacturer. A *nptII* antisense RNA probe was synthesized with pGEMnpt as a template (30). RNA size markers were purchased from BRL. DNA samples (10 μg) were digested using a 2-fold excess of restriction enzyme; after a 3-hr incubation, fresh enzyme was added, and digestion continued for 3 hr. Digested DNA was separated in 0.8% agarose gels using *Pst* I-digested phage λ DNA as a molecular mass marker. DNA was transferred to Hybond-N membranes (Amersham). Blotting and hybridization were done according to the manufacturer's instructions. DNA probes were generated with a megaprime DNA-labeling system (Amersham).

Run-On Transcription. The leaf tissue was harvested simultaneously for nuclei isolation and transcription reactions (19) and for DNA/RNA preparations. RNA isolation from nuclei with omission of the NaOH treatment, prehybridization, hybridization, and washing was as described (31). Linearized plasmid DNA (100 ng) containing gene-specific sequences was denatured and bound to nitrocellulose membranes (Hybond-C Extra, Amersham) by using a Hybri-Slot manifold (BRL). The weak hybridization signal seen for the

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Abbreviations: T-DNA, transferred DNA; P35S, cauliflower mosaic virus 35S promoter; Pnos, nopaline synthase promoter.

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negative control pGEM2 is probably from cross hybridization because of limited polylinker sequence homology between the pGEM2 plasmid and the transcribed chimeric *nptII* gene.

PCR. DNA methylation patterns were studied by PCR and a variant of the *Hpa* II-PCR assay (32). DNA samples were digested as described for Southern blotting, extracted with phenol/chloroform, and EtOH precipitated. Concentration of the resuspended DNA was determined with a spectrophotometer. DNA (0.75 μg) was incubated with 250 ng of primer (P1 and P2, P3 and P4, or P5 and P6) in 1 × *Taq* polymerase incubation buffer (Boehringer Mannheim); 2.5 units of *Taq* polymerase (Boehringer Mannheim) was added to a final 50-μl vol. Samples were heated to 94°C for 2–3 min before PCR. Denaturation was at 94°C for 45 sec, annealing was at 65°C for 45 sec, and extension was at 72°C for 1 min. Amplified fragments were separated on 2.0% agarose gels beside *Pst* I-digested phage λ DNA. When comparing methylation patterns of DNA isolated from normal and silenced plants, we found in a time-course analysis that amplification for 25 and 30 cycles yielded optimal diagnosis of the methylation status for the 3′ region and the promoter region, respectively. When the 183-bp control fragment and the 797-bp fragment were amplified in the same reaction, accumulation of the latter fragment was selectively reduced. Therefore, target DNAs were amplified separately and combined before loading on the gel. All experiments were done at least twice, and the accumulation profiles under these conditions proved reproducible. For location of primers P1, P2, P3, and P4 see Fig. 5; P5 and P6 are located in the coding region of the *hpt* gene that is not cut by any of the restriction enzymes in the PCR analysis. Sequence of the primers from 5′ to 3′ is as follows: P1, d(CAGGACATAGCGTTGGCTACCCGTG); P2, d(CCAATATAGCTCACATGCAGCACAC); P3, d(CCAGTATGGACGATTCAAGGCTTGC); P4, d(CCCCTGCGCTGACAGCCGGAACACG); P5, d(GCTTTGGGCCGAGGACTGCCCCGAAG); and P6, d(CTCCATACAAGCCAACCACGGCCTC).

RESULTS

Expression of a P35S–*nptII* Reporter Gene in Transformed Tobacco. Tobacco plants were transformed with a T-DNA construct harboring two chimeric genes: a *hpt* gene under control of the Pnos that was used as a selectable marker and the *nptII* reporter gene driven by P35S (Fig. 1). Most primary transformants accumulated high levels of *nptII* transcript (Fig. 2*A*). However, plants GVCHS(320)-1 and GVCHS-(320)-8 showed a substantially lower level of *nptII* steady-state mRNA. Segregation analysis suggested that GVCHS(320)-1 contained two independently segregating T-DNA loci (24 out of 340 germinating R1 seedlings were sensitive to hygromycin). Progeny plants obtained after self-fertilization of this primary transformant showed highly variable levels of *nptII* gene expression, ranging from the low level observed in the mother plant to the high level observed in most other primary transformants as well as intermediate levels (data not shown). From these progeny plants, two were selected with low *nptII* gene expression, R1-1 and R1-2 (hereafter designated silenced), and two were selected that had gained high *nptII* gene expression, R1-3 and R1-4. The level of *nptII* steady-state mRNA in plants R1-3 and R1-4 is

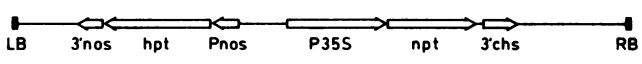

FIG. 1. Map of pGVCHS(320) T-DNA. 3′chs, 3′-Untranslated region of *An. majus* chalcone synthase gene; 3′nos, nopaline synthase 3′-untranslated region; hpt, hygromycin phosphotransferase; npt, neomycin phosphotransferase II; LB, left border; RB, right border.

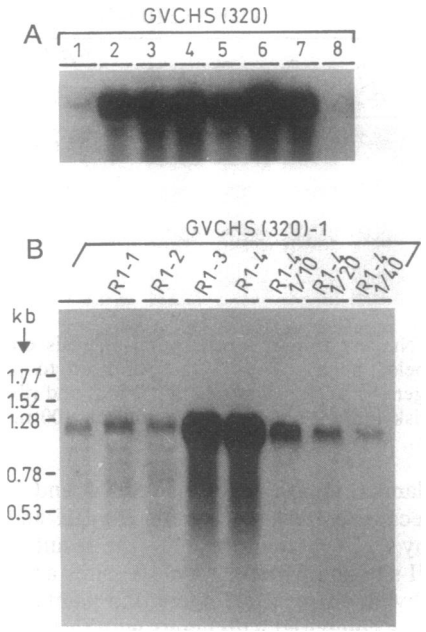

FIG. 2. (*A*) Northern blot analysis of the GVCHS(320) primary transformants. Hybridization was with a nptII RNA probe. Lane numbers correspond to numbers of individual plants. (*B*) Northern blot analysis of GVCHS(320)-1 (left lane) and progeny plants R1-1 through R1-4. Hybridization was with a nptII RNA probe. R1-4 RNA was 10-, 20-, and 40-fold diluted by using untransformed tobacco leaf RNA. RNA size markers are indicated at left.

20- to 40-fold higher than in the mother plant and the two progeny plants R1-1 and R1-2 (Fig. 2*B*).

Southern blot analysis revealed a complex pattern of integrated T-DNAs in the primary transformant GVCHS-(320)-1. The silenced R1-1 and R1-2 progeny plants had two T-DNA loci similar to those of the mother plant, whereas the R1-3 and R1-4 segregants had a single T-DNA locus that contained multiple *nptII* genes (data not shown).

Reduced Steady-State nptII mRNA Levels Are Not Associated with a Comparable Decrease in Transcription Initiation. To determine whether reduced steady-state nptII mRNA levels correlate with reduced transcription, run-on transcription analysis was done by using nuclei isolated from leaves. This assay measures the number of polymerase molecules actively engaged in transcription of the DNA template at the time of nuclei isolation. Transcription of the *nptII* transgenes and of the endogenous ribosomal (*rib*) and *rbcS* genes was assayed by hybridization of labeled nuclear RNA to plasmid DNA containing gene-specific sequences. Using the endogenous genes as internal controls, we found that the *nptII* genes are efficiently transcribed both in the silenced progeny plants and in the plants with high *nptII* gene expression (Fig. 3). Thus, the reduced steady-state nptII mRNA levels in plants R1-1 and R1-2 do not result from reduced transcription initiation. When R1-4 nuclei are incubated in the presence of α-amanitin at 2 μg/ml (R1-4+α-am.), transcription by RNA polymerase II (i.e., the *nptII* transgenes and the endogenous *rbcS* genes) but not by RNA polymerase I (the ribosomal genes) is inhibited, confirming the validity of the assay (Fig. 3).

Levels of *nptII* Gene Expression Are Inversely Correlated with *nptII* Gene Methylation. Plant DNA from the primary transformant and four progeny plants was digested with either *Bam*HI or *Hin*dIII and hybridized with probe 1 and 2, respectively (Fig. 4*A*). The Southern blots in Fig. 4 *B* and *C* show that the hybridizing bands in plants with high levels of *nptII* gene expression (Fig. 4 *B*, lanes 3 and 5, and *C*, lanes 4 and 5) are shifted to bands of higher molecular mass in the

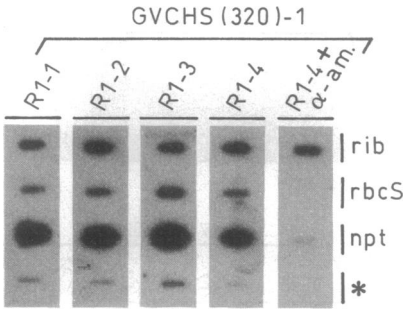

FIG. 3. Nuclear run-off transcription analysis on R1 progeny plants. Labeled nuclear RNA was hybridized to plasmid DNA containing gene-specific sequences: *npt*, *rbcS*, and ribosomal DNA (*rib*). Asterisk indicates the negative control, 100 ng of pGEM-1 DNA. α-am., α-Amanitin.

silenced plants (Fig. 4 *B*, lanes 1, 2, and 4, and *C*, lanes 1, 2, and 3). Because cleavage by both *Hin*dIII and *Bam*HI is inhibited by DNA methylation (33), the results suggest that the *Hin*dIII site and particular *Bam*HI sites are more extensively methylated (hypermethylated) in plants with reduced expression as compared with plants with restored *nptII* gene expression.

Hybridization of *Bam*HI-digested plant DNA to probe 1 shown in Fig. 4*A* should result in a 1.0-kb internal fragment and a number of T-DNA/plant DNA junction fragments. In addition to one junction fragment of ≈0.8 kb (fragment A), the 1.0-kb signal (fragment B) is the predominant signal in plants R1-3 and R1-4 (Fig. 4*B*, lanes 3 and 5). In contrast, the internal fragment B and the junction fragment A are barely detectable in the mother plant and the silenced progeny plants R1-1 and R1-2. Instead, two bands of higher molecular mass (a 1.8-kb and a 4.4-kb signal) are most prominent. We can putatively allocate these bands if we assume that the three *Bam*HI sites downstream from the *nptII* gene are

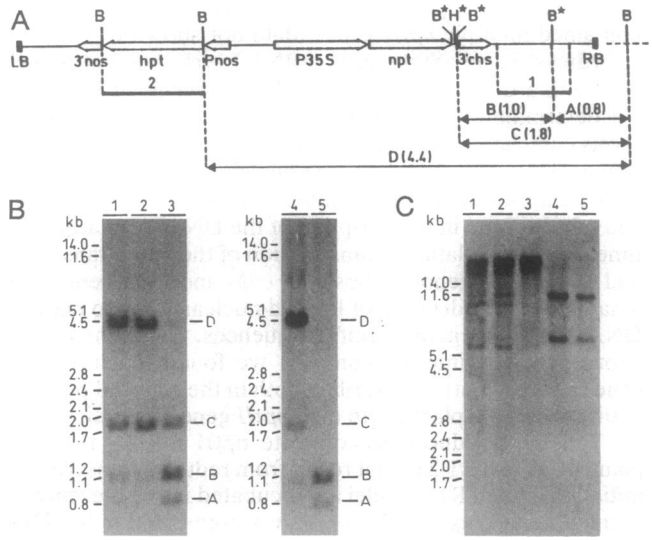

FIG. 4. Southern blot analysis on GVCHS(320)-1 and progeny plants R1-1 through R1-4. (*A*) Schematic representation of GVCHS(320) T-DNA and flanking sequences. Probes 1 and 2 are indicated by solid lines. B, *Bam*HI; H, *Hin*dIII. Fragments A, B, C, and D correspond to the signals shown in *B*. Modified sites are indicated with a star. (*B*) Southern blot of *Bam*HI-digested DNA. Hybridization was with probe 1. Lanes 1–5 contain DNA isolated from GVCHS(320)-1 and progeny plants R1-1, R1-3, R1-2, and R1-4, respectively. (*C*) Southern blot of *Hin*dIII-digested DNA. Hybridization was with probe 2. Lanes 1–5 contain DNA isolated from GVCHS(320)-1 and the progeny plants R1-1, R1-2, R1-3, and R1-4, respectively.

methylated. Additional Southern blot analysis indicated that the *Bam*HI site located in between the *hpt*-coding sequence and the Pnos is not modified in the silenced plants (data not shown). The hybridization signals of ≈1.8 kb and 4.4 kb correspond most probably to fragments C and D (Fig. 4*A*). The fact that a fragment of 3.6 kb does not occur among the partial fragments suggests that the *Bam*HI site close to the right border is more extensively methylated than the two *Bam*HI sites immediately downstream from the *nptII*-coding sequence.

Similar results were obtained with *Hin*dIII-digested plant DNA. Fig. 4*A* shows that a unique *Hin*dIII site is located in between the two *Bam*HI sites of the *nptII* chimeric gene. This site is completely digested in plants R1-3 and R1-4 but is only partially digested in the silenced plants (Fig. 4*C*). When this DNA blot was probed again with the endogenous *rbcS* gene, an identical hybridization pattern was obtained in the five lanes, demonstrating that the plant DNA was completely digested in all cases (data not shown).

Methylated Cytosines Do Not Necessarily Belong to the Symmetrical Motifs CG or CXG and Occur Upstream and Downstream from the *nptII* Coding Sequence. To confirm that the *Bam*HI partial digests are due to the presence of 5-methylcytosine, plant DNA was digested by the isoschizomeric restriction endonuclease pair *Mbo* I and *Sau*3A and subjected to PCR analysis. The procedure is based on the assumption that, when using DNA digested by a methylation-sensitive enzyme, the target DNA will be amplified only if the site(s) located in between the two primers is (are) methylated (see *Materials and Methods*; ref. 12). *Sau*3A and *Bam*HI are inhibited by methylation of the same cytosine, whereas *Mbo* I is insensitive to cytosine methylation (Fig. 5*A*). *Sau*3A and *Mbo* I also show differential sensitivity toward N^6-methyladenine. Although this DNA modification occurs in some plant cells (34), it cannot account for the partial *Bam*HI digests because cleavage by *Bam*HI is not affected by N^6-methyladenine (30) (Fig. 5*A*). First, we analyzed the segment located in between the *nptII*-coding sequence and the 3′*chs* region that contains two *Bam*HI sites and an additional *Sau*3A site (Fig. 5*B*). The PCR analysis of *Sau*3A- and *Mbo* I-digested DNA shows that the three *Sau*3A sites are hypermethylated in the silenced *nptII* genes of plant R1-2 compared with the *nptII* genes that are normally expressed in plant R1-4 (Fig. 5*C*). Second, the region upstream from the *nptII*-coding sequence that contains four *Sau*3A sites, two in the promoter and two in the leader (Fig. 5*D*), was evaluated for methylation. Our results show that these sites are similarly hypermethylated in the silenced R1-2 *nptII* genes compared with the *nptII* genes normally expressed in plant R1-4 (Fig. 5*E*). We can conclude that in plant R1-2 methylated cytosines occur in the region upstream and downstream from the *nptII*-coding sequence.

DISCUSSION

The described transgene silencing is clearly a case of cosuppression: the P35S-driven transgenes in one of the T-DNA loci exert silencing and methylation upon homologous transgenes in another locus (in trans). Characteristically, the mRNA levels of cosuppressed transgenes are reduced 20- to 40-fold as compared with normal expression levels. The observed cosuppression is associated with increased DNA methylation. Release of the silencing by independent assortment markedly decreases methylation. Importantly, this *de novo* methylation is not restricted to cytosines located in the symmetrical CG or CXG context. We found that five out of nine analyzed 5-methylcytosines are not located in the CG or CXG context. Most strikingly, the reporter-gene silencing is not due to reduced transcription initiation, as would be expected, but it is mediated by a posttranscriptional event.

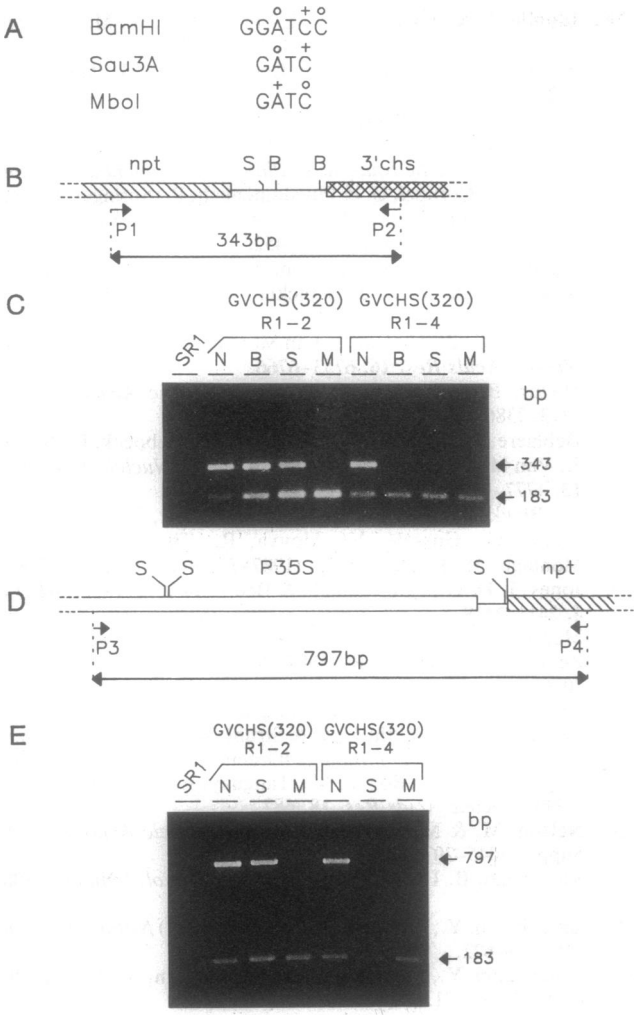

FIG. 5. PCR analysis of methylation in different regions of *nptII*. (*A*) Recognition sequence of *Bam*HI, *Sau*3A, and *Mbo* I. Å and Ĉ indicate the inhibition of cleavage by N^6-methyladenine and 5-methylcytosine, respectively. Å and Ĉ indicate that cleavage is not influenced by methylation. (*B*) Schematic representation of the region of the *nptII* chimeric gene that contains the two putatively modified *Bam*HI sites. Positions of *Bam*HI (B), and *Sau*3A/*Mbo* I (S) sites and primers P1 and P2 are indicated. (*C*) PCR analysis. Lanes: SR1, nondigested DNA isolated from untransformed tobacco plants; N, nondigested DNA. Lanes B, S, and M indicate *Bam*HI, *Sau*3A, and *Mbo* I digests, respectively. The 343-bp fragment corresponds to that shown in *B*. A 183-bp fragment located in the *hpt* gene was amplified as a control. The 343-bp and 183-bp fragments are recovered for both the silenced and highly expressing plant when nondigested DNA is used. When plant DNA is digested with *Bam*HI, a 343-bp band is present only for the silenced plant; similar results are obtained with *Sau*3A-digested DNA. However, when using *Mbo* I-digested DNA, no 343-bp fragment is amplified. This result indicates that these *Sau*3A sites are hypermethylated in the silenced plant R1-2 but are not hypermethylated in the plant with high *nptII* expression, R1-4. (*D*) Schematic representation of the promoter proximal region of the *nptII* chimeric gene. Positions of *Sau*3A/*Mbo* I sites (S) and primers P3 and P4 are indicated. (*E*) PCR analysis. For abbreviations, see *C*. The 183 bp is the *hpt* control fragment; 797 bp corresponds to the fragment shown in *D*. The 797-bp fragment is amplified for nondigested DNA as well as for the *Sau*3A digest of the silenced plant but is not amplified for the *Mbo* I digests or for the *Sau*3A digest of the plant with high *nptII* expression. This result indicates that these *Sau*3A sites are hypermethylated in the silenced *nptII* genes and hypomethylated in *nptII* genes that are highly expressed.

Cytosine methylation is known to occur principally at the sequence CG in animal cells (35) and at CG or CXG in higher plant cells (36). These sequences have strand symmetry, and there is strong experimental evidence that in higher eukaryotes this symmetry is implicated in maintaining methylation patterns after DNA replication (35). Methylation of cytosines located in non-CG or non-CXG sequences requires *de novo* methylation and has been found associated with the processes of rearrangement induced premeiotically in *Neurospora crassa* (37) and of methylation induced premeiotically in *Ascobolus immersus* (38), both of which cause silencing of repeated gene sequences. Extensive methylation of cytosine residues that are not in a symmetrical context has also been found in plants (39–43). These data (37–43) raise the question whether C-methylation in a nonsymmetrical context is characteristic of particular silencing phenomena both in fungi and in higher plant cells. Gene silencing of repeated sequences in fungi by rearrangement induced premeiotically and methylation induced premeiotically is a reciprocal event and occurs pairwise (44). In addition, the DNA methylation associated with the methylation-induced-premeiotically gene-silencing process is coextensive with the length of the repeats (45), and restored gene expression is associated with reduced methylation. Together, the observations support the view that gene silencing and methylation in fungi require a direct interaction between the duplicated sequences via ectopic pairing (for review, see ref. 46).

In maize, the activity of mutator and activator transposons (39–42) is inversely correlated with extensive methylation of both CG and non-CG cytosine residues. In this respect, an important finding is the coordinate epigenetic loss of *Mu* activity and the acquisition of methylation in two unlinked loci as a cell-autonomous clonal event, suggesting an unidentified variegating factor acting in trans. In petunia, the transcriptional silencing of a paramutated transgene has recently been shown to correlate with extensive cytosine methylation, which was absent in the adjacent T-DNA gene (12, 43). In all cases described above, however, the silencing and methylation seem to be meiotically heritable. Only a minority of the progeny cells contain genes that return to an active and unmethylated phase. Moreover, all the above examples correlate with transcriptional inactivation. Here, we report on extensive C-methylation that is readily meiotically reversible upon segregation of a silencing locus and is, nevertheless, posttranscriptional.

The nuclear run-on assays show that the 20- to 40-fold reduced steady-state nptII mRNA levels are not associated with an equivalent decrease in transcription initiation. However, it is a generally accepted rule that promoter methylation results in silencing by transcriptional control. As shown for several methylation-mediated silencing events in animal cells (47–50), transcription initiation could be inhibited because specific sequences involved in transcription factor binding are methylated. However, we consistently found equal amounts of nptII nuclear run-on transcripts of normally expressing and of silenced and methylated *nptII* genes. The PCR analysis showed that, at least in the R1-2 progeny plant, several cytosines located in the 5′-untranscribed region were methylated. However, we do not know whether this is also the case for the transcriptional control sequences of P35S.

How this posttranscriptional gene silencing is established is unclear. Gene silencing in *A. immersus* was in some cases associated with the presence of truncated transcripts (45). RNA analysis suggested that in these cases silencing was due to premature transcription termination at the region of DNA methylation. By assaying run-on transcripts homologous to the *nptII* 3′ region, we could conclude that transcription proceeded several hundred bases beyond the methylated *Bam*HI and *Hin*dIII sites (data not shown). Moreover, the transcription profile for silenced genes and genes with normal expression levels was very similar, suggesting that transcription along the silenced *nptII* genes was normal. The observed methylation may, therefore, be considered as the result of

RNA-directed methylation, as shown by Wassenegger *et al.* (51). Rather than inhibiting the transcription initiation or elongation, the methylation may be involved in a switch of chromatin structure, leading to unproductive transcripts. The posttranscriptional silencing implies a specific RNA-degrading activity, which has long been thought to be located in the nucleus. However, this view has been challenged by Lindbo *et al.* (21), who propose that a cytoplasmic mRNA-degrading activity may function in some examples of cosuppression (21). Thus, the methylation in the nucleus and the RNA degradation in the cytoplasm may result from two different pathways in nucleus and cytoplasm, both triggered by a common mechanism.

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1. Napoli, C., Lemieux, C. & Jorgensen, R. (1990) *Plant Cell* **2**, 279–289.
2. van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N. M. & Stuitje, A. R. (1990) *Plant Cell* **2**, 291–299.
3. Jorgensen, R. (1992) *AgBiotech* **4**, 265N–273N.
4. Kooter, J. M. & Mol, J. N. M. (1993) *Curr. Opin. Biotechnol.* **4**, 166–171.
5. Matzke, M. & Matzke, A. J. M. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 53–76.
6. Flavell, R. B. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3490–3496.
7. Hobbs, S. L. A., Kpodar, P. & DeLong, C. M. O. (1990) *Plant Mol. Biol.* **15**, 851–864.
8. Hobbs, S. L. A., Warkentin, T. D. & DeLong, C. M. O. (1993) *Plant Mol. Biol.* **21**, 17–26.
9. Linn, F., Heidmann, I., Saedler, H. & Meyer, P. (1990) *Mol. Gen. Genet.* **222**, 329–336.
10. Mitteltsen Scheid, O., Paszkowski, J. & Potrykus, I. (1991) *Mol. Gen. Genet.* **228**, 104–112.
11. Assaad, F. F., Tucker, K. L. & Signer, E. R. (1993) *Plant Mol. Biol.* **22**, 1067–1085.
12. Meyer, P., Heidmann, J. & Niederhof, I. (1993) *Plant J.* **4**, 89–100.
13. Matzke, M. A. & Matzke, A. J. M. (1991) *Plant Mol. Biol.* **16**, 821–830.
14. Matzke, M. A., Primig, M., Trnovsky, J. & Matzke, A. J. M. (1989) *EMBO J.* **8**, 643–649.
15. Goring, D. R., Thomson, L. & Rothstein, S. J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1770–1774.
16. Hart, C. M., Fischer, B., Neuhaus, J.-M. & Meins, F., Jr. (1992) *Mol. Gen. Genet.* **235**, 179–188.
17. Brusslan, J. A., Karlin-Neumann, G. A., Huang, L. & Tobin, E. M. (1993) *Plant Cell* **5**, 667–677.
18. Neuhuber, F., Park, Y.-D., Matzke, A. J. M. & Matzke, M. (1994) *Mol. Gen. Genet.* **244**, 230–241.
19. Mol, J., Van Blokland, R. & Kooter, J. (1991) *Trends Biotechnol.* **9**, 182–183.
20. de Carvalho, F., Gheysen, G., Kushnir, S., Van Montagu, M., Inzé, D. & Castresana, C. (1992) *EMBO J.* **11**, 2595–2602.
21. Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. & Dougherty, W. G. (1993) *Plant Cell* **5**, 1749–1759.
22. Bollmann, J., Carpenter, R. & Coen, E. S. (1991) *Plant Cell* **3**, 1327–1336.
23. de Carvalho, F., Boerjan, W., Ingelbrecht, I., Depicker, A., Inzé, D. & Van Montagu, M. (1994) in *Plant Molecular Biology: Molecular Genetic Analysis of Plant Development and Metabolism*, eds. Coruzzi, G. & Puigdomènech, P. (Springer, Berlin), North Atlantic Treaty Organization-Advanced Science Institute, Ser. H, Vol. 81, pp. 437–452.
24. Ingelbrecht, I. L. W., Herman, L. M. F., Dekeyser, R. A., Van Montagu, M. C. & Depicker, A. G. (1989) *Plant Cell* **1**, 671–680.
25. Simoens, C. R., Gielen, J., Van Montagu, M. & Inzé, D. (1988) *Nucleic Acids Res.* **16**, 6753–6766.
26. Mazur, B. J. & Chui, C.-F. (1985) *Nucleic Acids Res.* **13**, 2373–2386.
27. Deblaere, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M. & Leemans, J. (1985) *Nucleic Acids Res.* **13**, 4777–4788.
28. De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, R., Thompson, C., Van Montagu, M. & Leemans, J. (1987) *EMBO J.* **6**, 2513–2518.
29. Jones, J. D. G., Dunsmuir, P. & Bedbrook, J. (1985) *EMBO J.* **4**, 2411–2418.
30. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
31. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1987) *Current Protocols in Molecular Biology* (Greene and Wiley, New York).
32. Singer-Sam, J., LeBon, J. M., Tanguay, R. L. & Riggs, A. D. (1990) *Nucleic Acids Res.* **18**, 687.
33. Nelson, M. & McClelland, M. (1991) *Nucleic Acids Res.* **19**, Suppl., 2045–2071.
34. Vanyushin, B. F. (1984) *Curr. Top. Microbiol. Immunol.* **108**, 99–114.
35. Gruenbaum, Y., Cedar, H. & Razin, A. (1982) *Nature (London)* **295**, 620–622.
36. Gruenbaum, Y., Stein, R., Cedar, H. & Razin, A. (1981) *FEBS Lett.* **124**, 67–71.
37. Selker, E. U. (1990) *Annu. Rev. Genet.* **24**, 579–613.
38. Rhounim, L., Rossignol, J.-L. & Faugeron, G. (1992) *EMBO J.* **11**, 4451–4457.
39. McCarty, D. R., Carson, C. B., Lazar, M. & Simonds, S. C. (1989) *Dev. Genet.* **10**, 473–481.
40. Schwarz, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2789–2793.
41. Walbot, V. (1992) *Mol. Gen. Genet.* **234**, 353–360.
42. Martienssen, R. & Baron, A. (1994) *Genetics* **136**, 1157–1170.
43. Meyer, P., Niedenhof, I. & ten Lohuis, M. (1994) *EMBO J.* **13**, 2084–2088.
44. Faugeron, G., Rhounim, L. & Rossignol, J.-L. (1990) *Genetics* **124**, 585–591.
45. Barry, C., Faugeron, G. & Rossignol, J.-L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4557–4561.
46. Rossignol, J.-L. & Faugeron, G. (1994) *Experientia* **50**, 307–317.
47. Iguchi-Ariga, S. M. M. & Schaffner, W. (1989) *Genes Dev.* **3**, 612–619.
48. Becker, P. B., Ruppert, S. & Schütz, G. (1987) *Cell* **51**, 435–443.
49. Boyes, J. & Bird, A. (1991) *Cell* **64**, 1123–1134.
50. Boyes, J. & Bird, A. (1992) *EMBO J.* **11**, 327–333.
51. Wassenegger, M., Heimes, S., Riedel, L. & Sänger, H. L. (1994) *Cell* **76**, 567–576.